0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(5):2273–2279

doi: 10.1210/en.2005-1565

Starting the Zebrafish Pineal Circadian Clock with a Single Photic Transition

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The issue of what starts the circadian clock ticking was addressed by studying the developmental appearance of the daily rhythm in the expression of two genes in the zebrafish pineal gland that are part of the circadian clock system. One encodes the photopigment exorhodopsin and the other the melatonin synthesizing enzyme arylalkylamine *N*-acetyltransferase (AANAT2). Significant daily rhythms in AANAT2 mRNA abundance were detectable for several days after fertilization in animals maintained in a normal or reversed lighting cycle providing 12 h of light and 12 h of dark. In contrast, these rhythms do not develop if animals are maintained in constant lighting or constant darkness from fertilization. In contrast to exorhodopsin, rhythmicity of AANAT2 can be initiated by a pulse of light against a background of constant darkness, by a pulse of darkness against a background of constant lighting, or by single light-to-dark or dark-to-light transitions. Accordingly, these studies indicate that circadian clock function in the zebrafish pineal gland can be initiated by minimal photic cues, and that single photic transitions can be used as an experimental tool to dissect the mechanism that starts the circadian clock in the pineal gland. (*Endocrinology* 147: 2273–2279, 2006)

LIFE ON EARTH is characterized by a 24-h pattern of activity, reflecting the solar day. In essentially all forms, these rhythms are not directly controlled by the light/dark cycle, but reflect the action of an endogenous timing mechanism—the circadian clock—which exhibits an approximately 24-h period. In addition, mechanisms exist through which the environmental lighting cycle synchronizes circadian rhythms with light-to-dark and dark-to-light transitions.

In contrast to the significant progress made in understanding the molecular basis of circadian clocks and how light acts on the clock (1, 2), scant progress has been made in understanding the fundamental question of what initially starts the clock—ticking—as defined by producing a coordinated output signal—and whether a photic perturbation is required. This issue is of interest and importance in view of the fundamental role that circadian rhythms play in biology.

The question of whether the vertebrate circadian clock function develops autonomously or requires an external trigger has been investigated in zebrafish, at the molecular, physiological, and behavioral levels. This has generated conflicting results. For example, studies on the transcription factor *zPer3,* a component of the circadian clock machinery, are at odds: expression was reported to oscillate under constant darkness (DD) by one group (3) but not by another using transgenic embryos expressing luciferase under control of the *zPer3* promoter (4). It is clear that conclusions regarding clock function cannot be based on observations of *zPer3* expression alone because zPer3 is just one among other components of the clock machinery; accordingly, it may not be a reliable indicator of the functional state of the clock. Other approaches in which physiological and behavioral endpoints have been used are also problematical because the endpoints are a reflection of factors other than clock development, including downstream processes and the mechanisms linking the two (5, 6). Accordingly, studies using these systems may not provide an entirely reliable indication of clock function.

An experimental approach that circumvents these limitations is the measurement of arylalkylamine *N*-acetyltransferase (AANAT2) mRNA in the fish pineal organ. The pineal organ is generally considered a master circadian clock in fish and is of special experimental value because each pinealocyte contains all elements of a circadian system—a photoreceptor, a clock, and an output (7), *i.e.* mRNA encoded by the zebrafish (*zf*)*AANAT2* gene. *Aanat* encodes the next-to-last enzyme in vertebrate melatonin synthesis and *zfAANAT2* expression in the adult is controlled by integral elements of the molecular circadian clock, thereby providing a reliable marker of clock function (8–11). In addition, measurement of zfAANAT2 mRNA, is superior to the measurement of melatonin release from intact embryonic zebrafish, which has been used to study clock function (5), because melatonin synthesis is regulated not only by the clock, but also by a

First Published Online February 23, 2006

Abbreviations: AANAT2, Arylalkylamine *N*-acetyltransferase; DD, constant darkness; dpf, days post fertilization; ExR, exorhodopsin; LD, light/dark; LL, constant light; zf, zebrafish.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

down-stream gating mechanism through which light acts to rapidly switch off melatonin production, without necessarily having immediate effects on clock function. As a result, it is not possible to study clock function in constant light, because light masks the output. However, measurement of zfAANAT2 mRNA eliminates this limitation because light does not have an immediate effect on this parameter.

In the current study, we have examined the relationship between lighting and the rhythmic expression of zfAANAT2 and of another clock-related gene, exorhodopsin (*ExR*), which encodes the photopigment thought to mediate photic control of the pineal clock (12). Our experiments addressed the issue of whether minimal photic perturbations can initiate circadian expression of each of these genes in the embryonic zebrafish pineal gland. Previous studies raised this possibility, because *zfAANAT2* mRNA transiently increased after exposure to a single 1-h light pulse against a background of constant darkness (12). The nature of the controlling mechanism was investigated here, to determine whether this increase was a transient one-time response to the light pulse (13) or represented the initiation of a circadian rhythm. The results establish that circadian rhythmicity is initiated by a single light pulse and extend this finding by revealing that rhythmicity can also be initiated by a single dark pulse or by single photic transitions (light-to-dark: L→D, or dark-to-light: D→L). Moreover, our findings indicate that clock function is initiated before the retina can detect light and at a time when the pinealocyte is anatomically immature.

Materials and Methods

Animals

Adult zebrafish were kept at 28.5 C on a light/dark (LD) cycle (12 h light, 12 h dark). Light was provided by fluorescent lamps (λ = 400–700 nm; Nominal TX Universal aquarium lamps (IRC70-Lumen/W70), Actizoo, Beaufort La Vallée, France); the intensity at tank level was 5000 lux. Fertilized eggs were usually obtained in the morning shortly after lights on. They were incubated at the same temperature and under the indicated photoperiodic conditions (see *Results* and figure legends). Embryos were collected at different developmental stages. Dark samples were maintained in the dark until time of collection; dim red light used for night collections was provided by 230V/15W red bulbs (PF712E; Philips, Suresnes, France), with an intensity not exceeding 6 lux at the level of the embryos.

For the whole-mount *in situ* hybridization, pigmentation of the embryos was prevented by addition of 0.2 mM phenylthiourea to the egg water at 24 h post fertilization (hpf).

Animal experimentation was conducted in accord with accepted standards of humane animal care (principles set out in the Declaration of Helsinki).

Whole-mount in situ hybridization

At the end of the incubation time, embryos were fixed overnight in 4% paraformaldehyde in PBS at 4 C, transferred to 100% methanol, and stored at −20 C.

Whole-mount *in situ* hybridization was performed using commercially available kits according to the manufacturer's instructions (Roche, Meylan, France) (11, 14). Sense and antisense digoxygenin-labeled *zfAANAT2* riboprobes corresponding to a 900-bp cDNA of *zfAANAT2* (GenBank accession no. AF124756) were generated as described (11). The digoxygenin-labeled *zfExR* probes were generated from a fragment corresponding to bp 28–602 from *zfExR* (GenBank accession no. AB025312) inserted in the Topo vector (Invitrogen, Meylan, France). All probes (1 μg/ml) were hybridized at a temperature of 68 C, and detection was performed using alkaline phosphatase-conjugated antidigoxigenin antibodies, and using alkaline phosphatase substrate (Roche, Meylan, France). For each embryo, intensity of the staining was submitted to triple evaluation by three independent observers, within a scale of 0 (no staining at all) to 4 (highest signal intensity) (3). Preliminary studies indicated that densitometry measurements on black and white photomicrographs gave similar results as also shown elsewhere (11).

Statistics and data plotting were performed using the Instat-3 and PRISM-4 softwares (GraphPad). Each time point included five to 10 individuals, and all embryos from a given experiment were treated simultaneously. Data are presented as the mean ± SEM; they were analyzed by one-way or two-way ANOVA. One-way-ANOVA was followed, when significant, by Tukey's *post hoc* comparison of means (for convenience, only a comparison of the higher and lower means are reported in the legend of the figures). Experiments were repeated at least twice, each on different days. Pictures were obtained using a C-35AD-2 Olympus camera on a BH-2 Olympus microscope (Rungis, France).

Electron microscopy

Embryos and larvae were sampled at 22, 48, and 72 hpf and 11 d post fertilization (dpf), and fixed for 2 h in 4% glutaraldehyde/2% paraformadehyde, in 0.1 M phosphate buffer (PB) pH 7.4. After a brief rinsing in PB, they were postfixed in 1% osmium tetroxide for 1 h at room temperature. After rinsing in PB, all specimens were dehydrated in a graded ethanol series and embedded in araldite. Ultra-thin (50–70 nm thick) sections made on the pineal area were stained with 7% uranyl acetate in methanol, and contrasted with lead citrate (15). Sections were viewed and photographed on a Hitachi 7500 transmission electron microscope (Elescience, Verrières, France).

Results

Appearance of photoreceptor characteristics in developing pinealocytes

The embryonic pineal organ expresses *ZfExR* starting at 18 hpf (Fig. 1A). The area of ExR expression becomes larger at 24 hpf (Fig. 1B). Expression is limited to the pineal organ until 72 hpf (Fig. 1, C–E); at 96 hpf weak expression occurs in the ventral retina (Fig. 1F) at the level of the photoreceptor layer (Fig. 1, G and H).

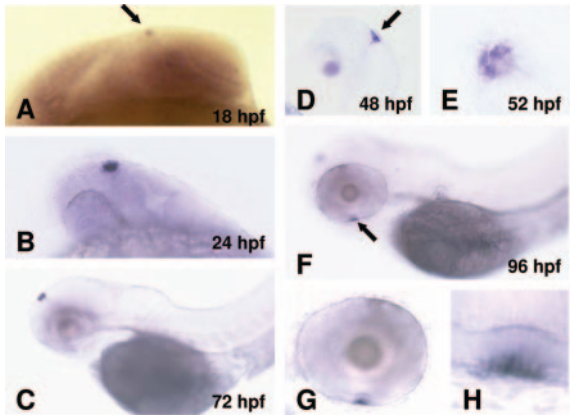

FIG. 1. Localization of ExR expression in the developing zebrafish embryo. ExR mRNA was visualized using *in situ* hybridization as indicated in *Materials and Methods*. A–E, With the antisense probe, a specific expression is evidenced in the pineal organ, which appears as a spot located dorsally of the embryo's head as early as 18 hpf (*arrow* in A). At higher stages, *i.e.* from 24 (B) to 72 (C) hpf, the spot appears located dorsally (D) in the midline of the head, between the eyes. E, Dorsal view of an embryo's pineal spot at 52 hpf; a few individual labeled cells are seen. F–H, At later stages, some expression also appears in the ventral retina as shown by the *arrow* in F. G and H, Higher magnifications of the labeled retina in F. The yolk in C and F shows weak nonspecific staining.

Electron microscopic examination revealed that the initial *zfExR* expression (18 hpf) is accompanied by the appearance of two defining structural characteristics of photoreceptors, centrosomes and [2 × 9 + 0] ciliary structures (Fig. 2A); a third feature of photoreceptors, the extensive infoldings of plasma membranes, first becomes obvious at 48 hpf (Fig. 2B).

Rhythmic expression of zfExR and zfAANAT2 develops in LD but not in constant light (LL) and DD

zfExR and zfAANAT2 mRNAs were studied in parallel as a function of environmental lighting. Daily rhythms in both were detected in LD, but were only statistically significant for zfAANAT2; rhythmic changes in these mRNAs were not apparent in LL or DD (Fig. 3).

Circadian rhythmicity of zfAANAT2 is maintained following LD cycles or a single dark pulse

zfAANAT2 was expressed rhythmically for at least 24 h in LL, if animals were first exposed to either two 12:12 LD (not shown) or two DL (Fig. 4) cycles. It was also found that rhythmicity was induced in LL after a single dark pulse applied at 24 hpf, and that the phase of the circadian rhythm was a function of pulse duration (Fig. 5). The finding that the zfAANAT2 mRNA rhythm persists for two cycles, and with similar amplitude, strengthens the idea that this represents a true circadian rhythm, rather than a one-time transient response.

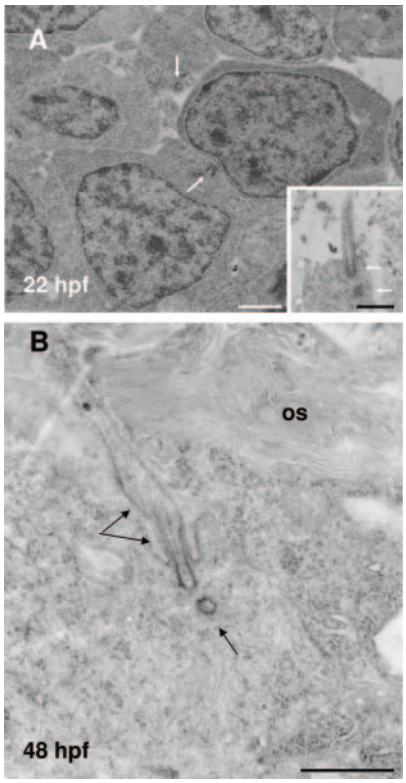

FIG. 2. Ultrastructure of zebrafish pineal photoreceptor cells at early stages of development. A, At 22 hpf, the pineal cells show little signs of differentiation, except the cilia of the 9 × 2 + 0 type and the associated centrosome (*arrows* and *inset*). B, At 48 hpf, photoreceptors are fully differentiated, and the well developed outer segment (OS) arises from the cilia (*arrows*). *Bars*, 1 μm.

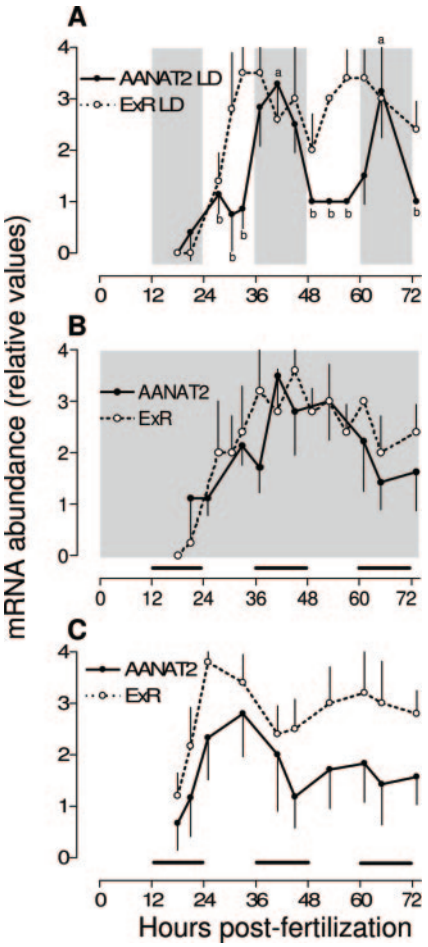

FIG. 3. zfExR and zfAANAT2 mRNA abundance under LD, DD, and LL conditions. Embryos were kept at 28.5 C under either a 12:12 LD cycle (A), DD (B) or LL (C). The *gray boxes* correspond to the dark phases (which are the same as those of the embryos genitors in A). The *black bars at the bottom* (in B and C) indicate the subjective dark phases (dark phases of the genitors). Embryos were collected at the times indicated and processed for the *in situ* hybridization; mRNA abundance was estimated as indicated in *Materials and Methods*. Mean ± SEM (n = 7–10). Zero in the abscissa corresponds to 0800 h (time of fertilization). One-way ANOVA indicated that AANAT2 mRNA abundance did not vary significantly under DD or LL. However, significant changes occurred under LD ($P < 0.0003$); a, significantly different from b (Tukey's *post hoc* comparison of means, $P < 0.05$). Differences in ExR mRNA abundance were not statistically significant under any of the conditions tested. Similar results were obtained in two other experiments.

Photic pulses initiate circadian rhythmicity of zfAANAT2

The above experiment used a dark pulse against an LL background. Similarly, a light pulse (1–8 h) against a DD background induced circadian expression of *zfAANAT2* (Fig. 6): the phases of the subsequent oscillations were similar, independent of the pulse duration. However, variations induced by the 1-h pulse were not significant and of lower amplitude than those obtained with the 2- or 8-h pulses.

Conversely, the appearance of the peak in AANAT mRNA abundance depended on the time at which the pulse was

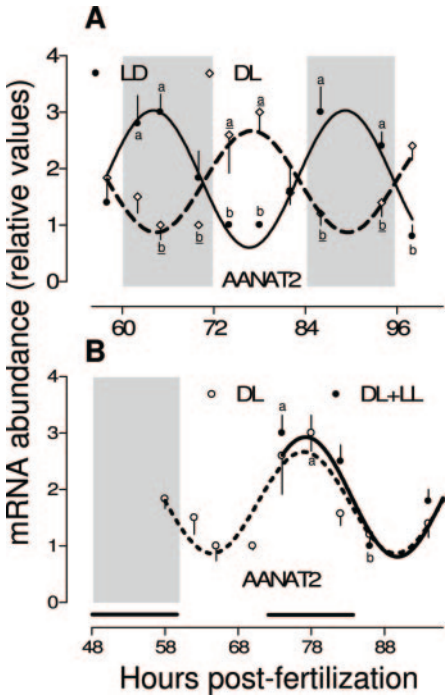

FIG. 4. zfAANAT2 mRNA abundance under a reversed DL cycle (A) followed by constant light (B). A, Embryos were placed under a LD cycle (controls; *solid line*) or a reversed DL cycle (DL; *dashed line*) from fertilization. B, An initial DL cycle was followed by LL at 60 *hpf (solid line)*; the controls (*dashed line*) were maintained under DL (same as DL cycle in A). The gray panels correspond to the dark phases. Embryos were collected at the times indicated and processed as in Fig. 3. Mean ± SEM (n = 7–10). The data fitted a sinusoidal; in each curve the variations in mRNA abundance were statistically significant (one-way ANOVA: $P < 0.005$ or below). a, Significantly different from b; *a*, significantly different from *b* (Tukey's *post hoc* comparison of means, $P < 0.05$). Similar results were obtained in two other experiments.

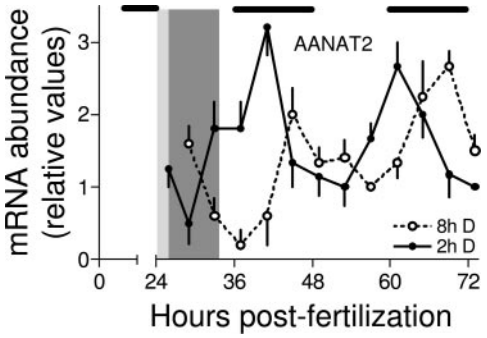

FIG. 5. Effects of dark stimuli of different durations on zfAANAT2 mRNA abundance in embryos maintained under LL. Embryos were maintained under LL from fertilization; a dark stimulus of 2 (*light gray*, *solid line*) or 8 h (*dark gray*, *dashed line*) duration was applied at 24 hpf, as indicated. Embryos were then processed as indicated in Fig. 3. The *black bars at the top* of each column indicate the subjective dark phases (dark phases of the genitors). Mean ± SEM (n = 7–10). In each curve, the observed variations in mRNA abundance were statistically significant as indicated by the one-way ANOVA analysis ($P < 0.005$); Tukey's *post hoc* comparison of individual means indicated that peak and nadir values differed from each other ($P < 0.05$). Pulse duration affected significantly the shape of the oscillations, as indicated by a two-way ANOVA comparison of the data ($P < 0.0008$). The 0 in the abscissa indicates time of fertilization (0800 h). Similar results were obtained in a second experiment.

applied. Thus, 4-h pulses of light applied at successive points during the 20- to 46-hpf period induced corresponding delays in peak appearance (Fig. 7).

Single photic transitions (D→L or L→D) initiate circadian rhythmicity of zfAANAT2

The above photic perturbations involve two photic transitions, L→D at the start and D→L at the end of the dark pulse. It was also found that a single cue initiated clock function when the D→L transition occurred at 21 hpf or later (Fig. 8, A–E). In a reversed experiment, embryos were raised under LL and then switched to DD; this initiated circadian oscillations when the L→D transition occurred at 21 hpf or later (Fig. 8A′–E′).

zfExR expression is not circadian

The expression of *zfExR* was examined in all the experiments described above. However, statistically significant rhythmic expression of *zfExR* did not occur.

Discussion

One of the advances of this study is finding that elements of the three functional components of circadian clock system in the zebrafish pineal organ—photodetection, clock function and output—appear to be in place at the end of 1 dpf. This is coincident with the appearance of the first pinealofugal nerve fibers (19 hpf) (16). It is of interest to note that at this point in development, pinealocytes appear to be anatomically immature compared with those observed d 2 (this study) or 6 dpf (17). At 24 hpf they lack the defining feature that characterizes the adult pinealocyte—distinct photoreceptor outer segments—while containing the characteristic $2 \times 9 + 0$ cilia from which the outer segments develop (17–19). The expression of *ZfExR* at 1 dpf is likely to explain pinealocyte photosensitivity at this stage. Accordingly, it appears that at 1 dpf, when the pinealocyte is not anatomically mature, it is fully capable of functioning as a complete circadian system, capable of responding to light. The interpretation that at this stage of development light acts indirectly on the pineal gland through another structure is less attractive because it assumes a developmental extrinsic→intrinsic switch in how light regulates the pineal circadian clock.

Our results also provide additional support to the view that fish pineal photoreceptors develop before retinal photoreceptors (20). Specifically, at 24 and 48 hpf, photodetector features were not evident in the zebrafish retina (data not shown). The first rudiments of retinal photoreceptor outer segments are not observable before 54 hpf (21), whereas the initial retinal expression of an opsin gene is not seen before 40 hpf (22). It is, however, not impossible that other pigments function during this period of time in the retina. We also detected *ZfExR* expression at 4 dpf in the ventral retina, as has been described for *Aanat2* (detectable at 3 dpf) (11), indicating that retinal *zfExR* expression is developmentally transient (11). In contrast to the 4 dpf retinal expression of zfExR, pineal expression was detectable at 24 hpf, when structural features of photoreceptors were also apparent. Earlier development of pineal photoreceptors relative to that

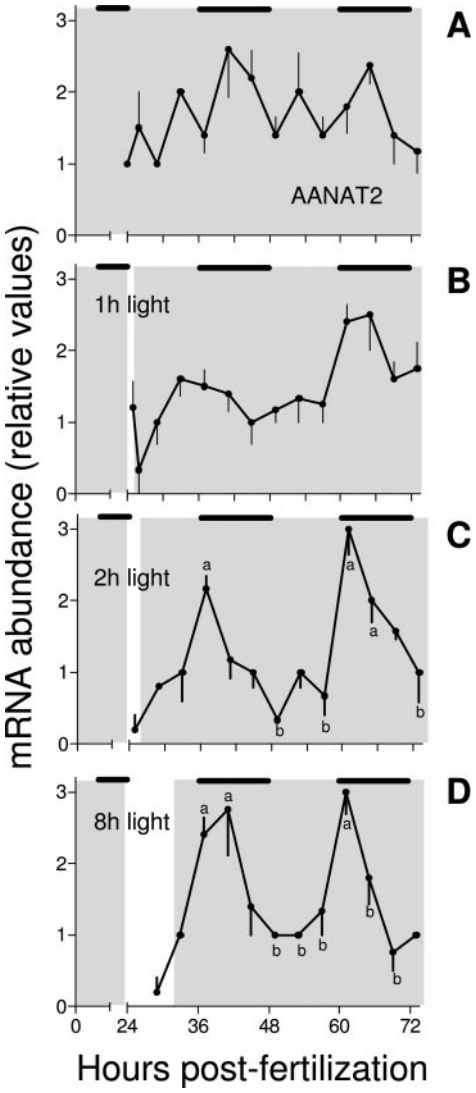

FIG. 6. Effects of light pulses of different duration on AANAT2 mRNA abundance in embryos maintained under constant darkness (DD). Embryos were maintained under DD (A) from fertilization and a light pulse of 1 (B), 2 (C) or 8 (D) h was applied at 24 hpf, as indicated. They were then processed as indicated in Fig. 3. The *black bars* at the *top* indicate the subjective dark phases (dark phases of the genitors). The *gray panels* indicate darkness for the embryos. Mean ± SEM (n = 7–10). The 0 in the abscissa indicates time of fertilization (0800 h). One-way ANOVA indicated that the variations in mRNA abundance were statistically significant only in C (2-h light pulse) and D (8-h light pulse) ($P < 0.0001$). a, Significantly different from b (Tukey's *post hoc* comparison of means, $P < 0.05$). Two-way ANOVA indicated that the 2- h and 8-h light pulses curves were significantly different from the DD curves ($P < 0.01$), but the duration of the pulse (2 h *vs.* 8 h) had no significant effect ($P < 0.7$). Similar results were obtained in another experiment.

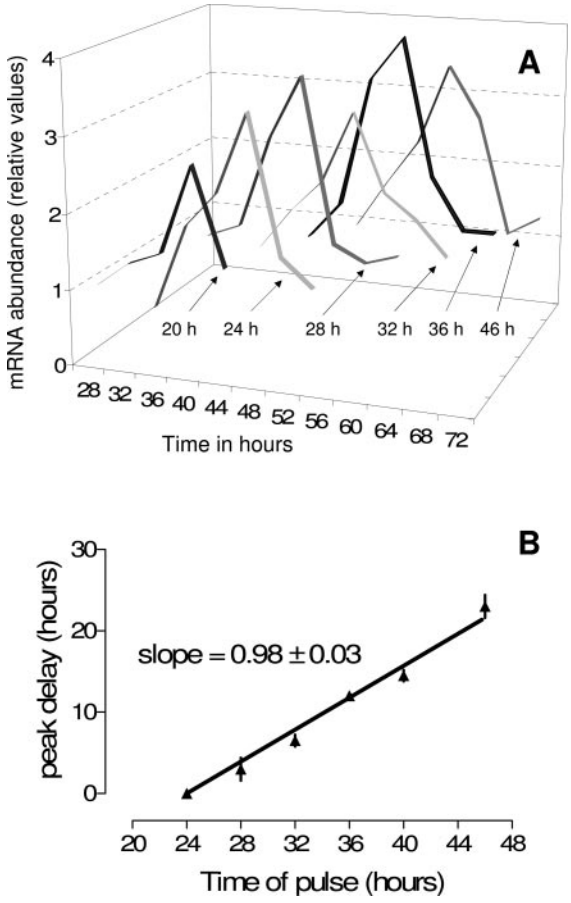

FIG. 7. Effects of light pulses applied at different post-fertilization times on the circadian expression of AANAT2 mRNA abundance in embryos maintained under DD. A, Embryos were maintained under DD from fertilization. A 4-h light pulse was applied at 24, 28, 32, 36, 40, or 46 hpf as indicated; embryos were collected shortly after at different time intervals and processed as indicated in Fig. 3. Mean ± SEM (n = 5–10); *error bar*s are not shown for clarity of the graph. ANOVA indicated that the variations in mRNA abundance differ significantly in all curves ($P < 0.0001$); in each curve, Tukey's *post hoc* comparison of means indicated that the peak values were different from the nadir values ($P < 0.001$). B, Data are replotted from those presented in A. There is strict correlation between the times at which the light pulses are applied (abscissa) and the corresponding phase delays in peak appearance reported in the ordinates (time of peak appearance minus time of peak obtained with the first pulse of light).

of retinal photoreceptors is intriguing, because similar transcription factors control expression of the same or closely related genes in each tissue. It will be of interest to determine the molecular basis of this temporal difference.

Previous observations have been interpreted to indicate that the nocturnal increase in zfAANAT2 mRNA abundance is itself triggered by light-induced expression of *zfPer2* (13). If this were the only condition, however, one would expect an increase in *zfAANAT2* expression to occur under LL once the pineal becomes activated, *i.e.* between 18 and 24 hpf. This was not observed, *i.e.* neither a surge, nor endogenous oscillations in *Aanat2* expression, was observed in embryos raised under LL from birth. Accordingly, it would appear more accurate to conclude that the embryos also needed to experience both dark and a photic transition. This is supported by the finding that circadian oscillations in *zfAANAT2* expression occurred under LL provided the embryos were previously exposed to an LD or DL cycle. Moreover, a single transition from DD to LL, or LL to DD, initiated circadian oscillations in *zfAANAT2*, providing that this transition occurred at 21 hpf or later. This is consistent with the proposal that exposure to both light and darkness is necessary to initiate pineal clock function. However, it is not yet clear

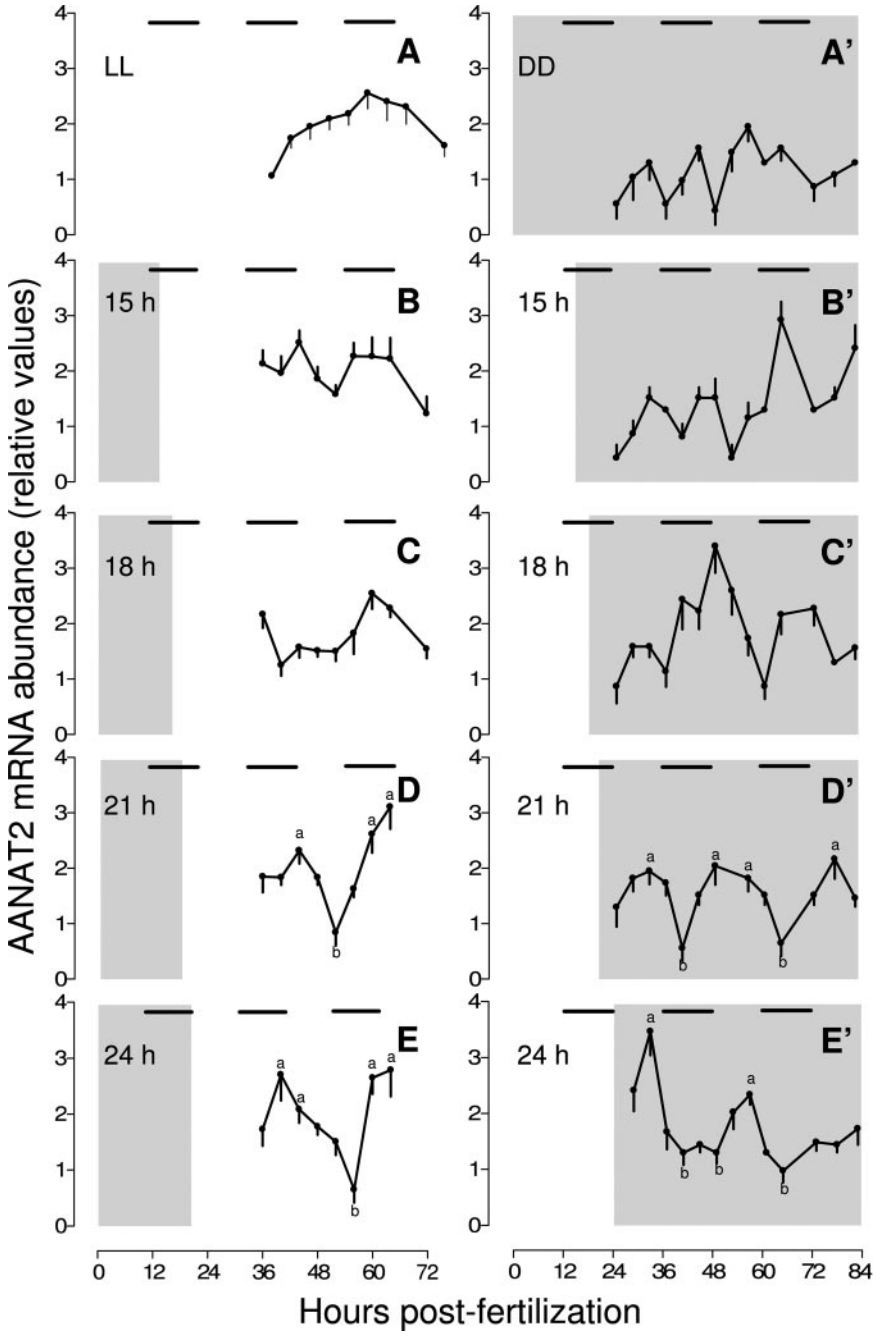

FIG. 8. Effects of dark-to-light and light-to-dark transitions on AANAT2 mRNA abundance. A–E, Embryos were maintained under constant light from fertilization (A: LL), or first exposed to darkness for 15 (B), 18 (C), 21 (D), or 24 (E) hours, before being released under LL. A′–E′, Embryos were first placed under constant darkness (A′, DD) from fertilization or exposed to light for 15 (B′), 18 (C′), 21 (D′), or 24 (E′) hours before being released under DD. They were then processed as indicated in Fig. 3. The *black bars* at the top of graph indicate the dark phases of the genitors, and the *gray boxes* indicate darkness for the embryos. Mean ± SEM (n = 7–10). Two-way ANOVA indicated that the curves in D and E were not different from each other, but differed from the others (A–C) ($P < 0.0001$). Similarly, the curves in D′ and E′ were not different from each other, but differed from the A′–C′ curves ($P < 0.0001$). a, Significantly different from b (Tukey's *post hoc* comparison of means, $P < 0.05$). The 0 in the abscissa indicates time of fertilization (0800 h). Similar results were obtained in an independent experiment.

whether this initiation of pineal clock function reflects the start of the circadian clocks in individual pinealocytes or the synchronization of active clocks in these cells, or both.

Synchronization of the clocks in individual pinealocytes may involve light-dependent induction of *zfPer2* (13), and dark-dependent induction of a yet-unidentified factor, both of which could interact with components of the transcriptional/translational feedback loop that constitutes the circadian clock (23, 24). From the effects of dark pulses under LL and light pulses under DD, it appears that the timing of light onset, not duration, determines the phase of the resulting circadian rhythm.

The results of these studies provide clear indication that *zfAANAT2* is under circadian control. This is likely to reflect the presence of E-Box elements (8), which mediate circadian control of many genes through interactions with protein components of the clock (25–27). The mechanism underlying the noncircadian photic control of *zfExR* expression in LD lighting cycles remains unknown.

In summary, the present study provides evidence that the circadian system in the zebrafish pineal organ is potentially functional as early as 18–21 hpf, *i.e.* 2 d before the retina. Anatomically immature pinealocytes are capable of transducing light signals and generating circadian oscillations in *zfAANAT2* expression. Accordingly, it appears that light sensitivity, circadian clock function, and output are present early

in embryonic development and that the pineal gland—not the retina—mediates photic responses at this stage. Our results also provide further support to the interpretation that minimal photic cues are sufficient to initiate pineal circadian clock function during development. Finally, it appears that light perception *per se*, as provided by LL, is not sufficient to initiate the circadian oscillations; rather, a photic transition is required, with the time of light onset determining the subsequent phase of the oscillations. This and previous studies (13) suggest that the onset and entrainment of the pineal circadian clocks need both light and darkness, *i.e.* light acting through induction of *zfPer2* expression, and darkness acting through a yet unknown mechanism. It remains to be determined whether the photic transitions (L→D or D→L) start the clock ticking in each cell or synchronizes circadian activity of individual cells, or does both. These questions may be asked, using the paradigm established here, in which single photic transitions are sufficient to generate a circadian output, *i.e.* zfAANAT2 mRNA.

Acknowledgments

The authors thank M. Fuentès, B. Rivière, and D. Saint-Hilaire for their technical assistance.

Received December 8, 2005. Accepted February 9, 2006.

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This work was supported by the Centre National de la Recherche Scientifique (Grant PICS 2556) and the Intramural Research Program, National Institute of Child Health and Human Development, National Institutes of Health.

Disclosure statement: R.V., L.B., G.B., A.P., Y.G., W.G.G., D.C.K., and J.F. have nothing to declare.

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